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APPLICATION REPORT
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New Proteomic Workflows Combine Albumin Depletion and On-Bead Digestion, for Quantitative Cancer Serum

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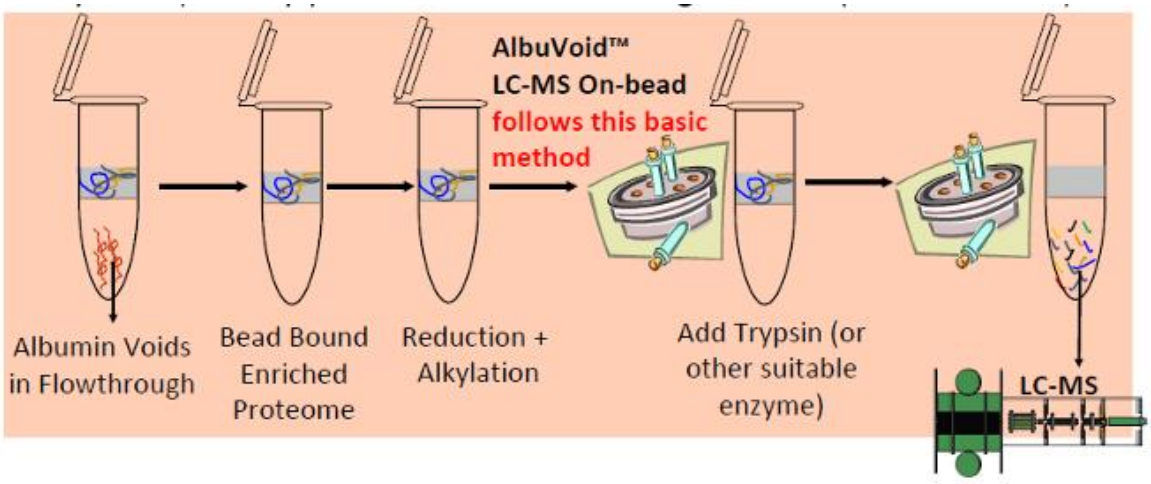
Introduction and Objectives

We demonstrate new workflows that support quantitative serum proteomic investigations and highlight several proteins that were observed to be up and down regulated from lung, breast and early stage pancreatic cancer sera. Proteomic workflows that support serum proteomics can be especially challenging for two reasons: 1) the presence of highly abundant proteins and 2) a particularly proteolytic resistant sample type due to the large post-translational carbohydrate presence.

For quantitative proteomics, having workflows that can greatly reduce one or more highly abundant proteins, along with short, efficient and consistent digestions will be highly desirable. Indeed several reports show that quantitative precision is strongly influenced by variations in enzymatic digestion efficiency[1-3]. We consider the efficiencies gained by an optimized workflow of albumin depletion, low abundance enrichment, and optimized on-bead digestions, to identify and quantify proteins from sera using isobaric (iTRAQ & TMT) labeling.



Methods

The workflow follows the **AlbuVoid™ LC-MS On-Bead** sample prep method[4]. In brief, 50 µl serum is prepared by adding a binding buffer, then applied to the **AlbuVoid™** beads, and washed. All steps are performed within a microfuge spin-filter format. Albumin and Transferrin are specifically voided out, while the low abundance serum proteome is retained on the bead. After the final wash, reduction, alkylation and Trypsin digestion all take place on the bead. The tryptic peptides were then labeled with iTRAQ (Sciex, Framingham, MA) in Experiment 1 and TMT6 (Proteome Sciences, Surrey UK) in Experiment 2. After labeling, the peptides were pooled and analyzed with a single LC-MS/MS 3 hour gradient run using nanoRSLC system interfaced with a Qexactive HF. MS data used data-dependent acquisition with resolution of 60,000 followed by MSMS scans (HCD 30% of collision energy) of 20 most intense ions with a repeat count of two and dynamic exclusion duration of 60 sec.

Experiment 1. In the first experiment, our goal was to observe quantitative differences between pooled lung and breast cancer patient sera relative to pooled normal sera. After overnight digestion, 201 total protein were identified and quantified, 20 of which were differentially observed, between the 3 serum samples. A complete list is available upon request. Table 1 is a list of the differentially observed proteins with 2 or more total spectral counts: Blue indicates up-regulated; green down-regulated.

Table 1 - differentially observed proteins Protein Identification [gene name]	Reporter Intensity Ratio Lung Cancer / Normal	Reporter Intensity Ratio Breast Cancer / Normal	Total Spectral Count
Immunoglobulin kappa variable [IGKV1-17]	1.2	5.5	4
Complement component 3 [C3]	3.3	3.2	325
S100 calcium binding protein A9 [S100A9]	2.3	2.4	2
serum amyloid A1 [SAA1]	3.8	2.0	13
serum amyloid A2 [SAA2]	3.8	2.0	43
CD109 molecule [CD109]	6.4	1.2	2
coagulation factor II (thrombin) [F2]	4.3	1.0	4
pro-platelet basic protein [PPBP]	2.7	0.9	12
BRF1, RNA polymerase III transcription [BRF1]	0.4	1.1	3
Fibrinogen alpha chain [FGA]	0.2	1.1	401
Fibrinogen beta chain [FGB]	0.2	1.1	141
Transthyretin [TTR]	0.4	0.5	7
sperm specific antigen 2 [SSFA2]	0.1	0.7	3
amyloid P component, serum [APCS]	0.4	0.5	4
immunoglobulin heavy cns delta [IGHD]	0.3	0.4	7

Experiment 1 Results and Discussion

While our quantitative goal was satisfied In Experiment 1, we only considered one digest time (overnight). Yet serum contains a high abundance of proteolytically resistant glycoproteins. In previous reports, we demonstrated that the on-bead digest time can impact the proteins and peptides identified from serum, producing three observable populations of proteins: i) proteins that are digested efficiently at short digestion times, but to which some peptides may be prone to non-specific digestion at long digestion times, or otherwise be masked by high proteolytic background, ii) proteins that are digested efficiently even at short digestion times, but whose observation is not negatively affected by long digestion times, and iii) proteins that require overnight digestion for proper identification[5]. Therefore, in the next experiment our goal was to study the impact of digest time on reproducibility and quantitative analysis, for cancer biomarker discovery applications.

Seeking Serum Protein Biomarkers for Early Stage Pancreatic Cancer

Experiment 2. Pancreatic cancer is usually diagnosed after clinical symptoms appear at which time it generally has a poor prognosis. As surgical resection is the only potentially curative treatment, to improve the prognosis of patients with pancreatic cancer, it is essential to detect tumors at very early stages, when they are resectable. New serum protein biomarkers that could be monitored over time could help the management of patients who are at an increased risk of familial pancreatic cancer.

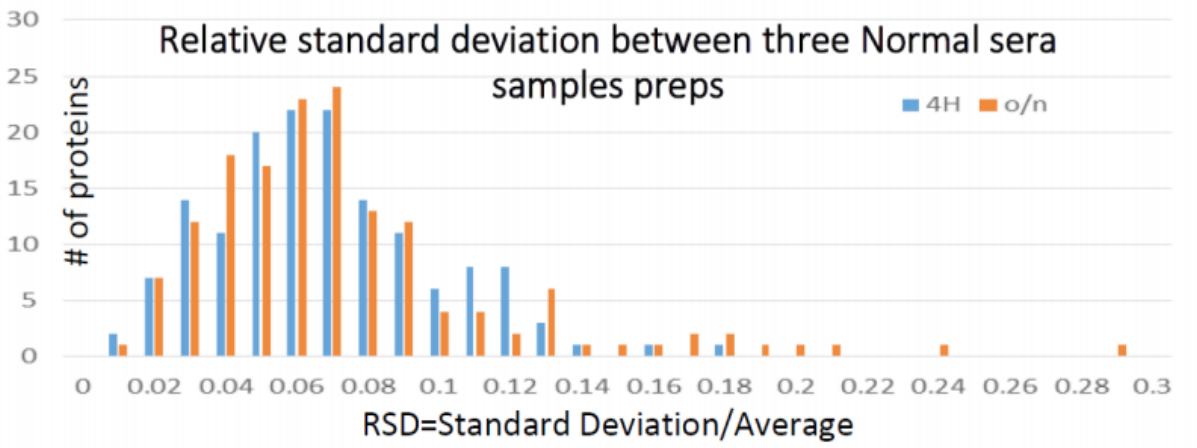
Methods were the same as in the first experiment with the exceptions that 2 digest times were performed and TMT-6 labels were used. The samples were commercially obtained (Discovery Life Sciences, Los Osos, CA) and are identified as:

N1, N2, N3: An individual normal sample split in 3 parts and prepared as triplicate **AlbuVoid™ LC-MS On-bead** in parallel methods.

P1: Individual patient 1 with early stage pancreatic cancer.

P2: Individual patient 2 with early stage pancreatic cancer.

P3: Individual patient 3 with stage 2 pancreatic cancer.



Experiment 2 Results and Discussion.

Proteins from both digest times (4 hours and overnight) were exceedingly reproducible, achieving our goal for this experiment. At the 4 hour digest time, all proteins from triplicate sample preparation (separations and on-bead digests) were quantified at <20% variance from average. The total number of proteins observed was 192; 170 at 4 hour digest, 182

at overnight digest, with 160 observed in both digest times. For quantitation, triplicate normal sample spectra were averaged and compared by ratio to the patient samples. Quantitative elements (spectral ratios and counts) were generally consistent between digest times and samples, with few but notable exceptions. A complete list of proteins and quantitation is available upon request. In Table 2, a selected panel of proteins with differential quantitation represent proteins considerate for future investigation.

Table 2 – Representative differentially observed proteins from pancreatic cancer samples

Digest Time	4 Hours				Overnight			
Protein ID [gene name]	P1/N	P2/N	P3/N	Spec Ct	P1/N	P2/N	P3/N	Spec Ct
Serum amyloid A protein [SAA2-SAA4]	1.4	1.7	1.6	15	1.0	1.9	2.0	10
Selenoprotein P [SEPP1]	0.8	0.3	0.4	9	0.8	0.5	0.5	18
Transthyretin [TTR]	3.3	0.5	0.8	6	1.6	0.9	0.9	3
Urea transporter 2 [SLC14A2]	0.0	0.6	0.4	3	0.0	0.3	0.2	3
Uncharacterized protein [C18orf63]					1.3	3.8	2.7	4
Dimethyladenosine transferase 1 [TFB1M]					0.2	0.2	0.2	3

Noteworthy is that Serum Amyloid A proteins are observed to over-expressed in all three cancers we tested and reported by others as over-expressed in lung cancer sera[6]. Also, Transthyretin has been reported to be under-expressed in both pancreatic and ovarian cancer sera[7,8]. We also observe it here as being differentially expressed, under-expressed mostly and in one case, over-expressed, in all three cancers. Selenoprotein P has also been observed as a potential biomarker in different cancers. One report concludes that its decreased concentration in serum might represent an additional valuable prostate cancer diagnostic[9]. Others suggest that a low plasma Selenoprotein P level is associated with higher future risk of respiratory and digestive tract cancer in middle-aged men[10]. It is interesting to consider that Serum Amyloid proteins along with Transthyretin and Selenoprotein P might be bellweather wellness proteins that become dysregulated during cancer progression. Urea transporter 2, Uncharacterized protein C18orf63, and Dimethyladenosine transferase 1 represent possible new serum biomarkers for early stage pancreatic cancer.

Conclusions

Using the new **AlbuVoid™ LC-MS On-bead** product, we spectrally quantified over 200 total proteins, many of which were differentially observed as over or under-expressed within the cancer sera compared to normal.

We suggest that it is practical and productive to evaluate different digestion times as this can impact both missed cleavages, and non-specific cleavages, which can bias protein quantitation. We solicit that the speed and efficiency of **AlbuVoid™ LC-MS On-Bead** digestions can minimize many of the inconsistencies of proteolytic hydrolysis improving reproducibility of serum or plasma peptides. This will prove advantageous for both quantitative discovery (shotgun) as well as targeted SRM/MRM applications.

Future Directions

> Investigate Serum Amyloid Proteins, Selenoprotein P, & Transthyretin as potential biomarkers for wellness, dysregulated with cancer progression.

> Investigate Urea Transporter 2, Uncharacterized Protein C18orf63, & Dimethyl Adenosine Transferase 1 as potential biomarkers for early stage pancreatic cancer

> Determine the individual variance of these same markers with respect to a pooled normal control, with ratio comparisons to normal individuals and early stage cancer patients.

> Develop a commercialization strategy to baseline monitor one or more of these same proteins, to offer high risk individuals better cancer management options.

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